AD_____

Award Number: W81XWH-09-1-0166

TITLE: Repression of TSC2 Expression by miR-296 in Human Normal and Tumor Tissues

PRINCIPAL INVESTIGATOR: Jian-Jun Wei

CONTRACTING ORGANIZATION: Northwestern University
Evanston, IL 60208

REPORT DATE: March 2010

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-03-2010	Final	1 Mar 2009 - 28 Feb 2010

4. TITLE AND SUBTITLE
Repression of TSC2 Expression by miR-296 in Human Normal and Tumor Tissues

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0166

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jian-Jun Wei

E-Mail: jianjun-wei@northwestern.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, IL 60208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Tuberous sclerosis complex (TSC) genes are tumor suppressors and are ubiquitous and expressed in almost all human tissues. Loss or inactivation of TSC genes contributes to a broad spectrum of diseases, including multiorgan neoplasia, and virtually any part of the body can be affected. Loss of the wild type allele and second somatic mutations is the major molecular mechanism identified in some but not all TSC-associated tumors. We proposed that microRNA miR-296 may be the native molecule regulating TSC2 expression during development and tumorigenesis in some TSC-associated tumors. To explore whether TSC2 can be regulated by the endogenous miRNAs during development and tumorigenesis, we will firstly study whether miR-296 is a native molecule that can specifically repress TSC2 expression in vitro. This can be accomplished by examination of TSC2 repression by exogenous miR-296 in cell models. Particularly we will examine whether presence of TSC2 3' untranslation region is critical for miR-296 regulation. We will then examine the expression correlation between TSC2 and miR-296 in human normal and tumor tissue samples. Identification of repression of TSC2 by microRNA miR-296 will provide a novel molecular mechanism of TSC gene regulations in development and TSC associated lesions. The findings will greatly impact on the differential diagnosis and potential therapeutic modality.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	8	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION

Tuberous sclerosis complex (TSC) genes are tumor suppressors and are ubiquitous and expressed in almost all human tissues. Loss or inactivation of TSC genes contributes to a broad spectrum of diseases, including multiorgan neoplasia, and virtually any part of the body can be affected. There are a few normal tissues and cell types (such as Glomeruli, pneumocytes, pituitary gland and thymus) that either do not express or express at an undetectable level of *TSC* genes or gene products. Genetic mutations of *TSC* genes are the initial events in familial TSC patients. To induce TSC-associated tumors, the second wild type allele must be inactivated. Loss of the wild type allele and second somatic mutations is the major molecular mechanism identified in all types of TSC-associated tumors, except for subependymal giant-cell astrocytomas (1) and rhabdomyomas (2). All of these findings suggest that there is(are) still another, more universal, mechanism(s) responsible for TSC-associated tumorigenesis.

Several alternative mechanisms that could potentially trigger TSC-associated neoplastic transformation have been proposed. AKT- and ERK-mediated inactivation of the TSC gene product (tuberin) is characterized to be a cellular mechanism leading to post translation modification of tuberin in TSC pathogenesis, even when one or both alleles of *TSC2* remains intact. However AKT-mediated tuberin inactivation seems to be less important, since the active AKT is not high in many TSC-associated tumors, such as subependymal giant-cell astrocytomas, angiomyolipomas, skin tumors and rhabdomyomas, but is at the same or even lower concentrations than healthy tissues (3). The role of pERK in regulating tuberin is still under active study. Epigenetic silencing of *TSC* genes is suggested but no methylation of the *TSC2* promoter region has been noted (4). Recently, it has been proposed that there may be a third protein in the TSC complex (putative TSC3) (5).

TSC-associated cardiac rhabdomyomas develop in the specific developmental stages (during the first years of life), and are not closely related to the germ cell or somatic cell TSC mutations (2). If TSC genes are indeed to play a critical role in this tumor, developmental regulation of TSC gene expression at the transcriptional and/or translational levels may exist. We proposed that microRNA (miRNA) *miR-296* may be the native molecule regulating TSC2 expression during development and tumorigenesis in some TSC-associated tumors. The hypothesis is largely based on the observations that: 1) differential expression of miRNAs at development can regulate cell proliferation and differentiation; 2) miRNA-mediated gene regulation is commonly associated with benign and malignant neoplasms; 3) there is a complementary site of *miR-296* at the TSC2 3' untranslation region (3'UTR), which is evolutionarily highly conservative (**Figure 1**); and 4) most importantly, there is an inverse association between tuberin and *miR296* expression, as observed in a pilot study of 36 smooth muscle tumors (unpublished data).

TSC2 contains 41 exons with a very short 3' untranslation region (3'UTR, <110 nt). The short *TSC2* 3'UTR may prevent from miRNA regulation. However, in the *TSC2* 3'UTR immediately adjacent to the stop code, there is a highly conserved sequence that harbors the complementary sites of *miR-296* and a few other miRNAs (**Figure 1**). *MiR-296* is over-expressed in many solid epithelial and mesenchymal neoplasms. In HeLa cells, inhibition of *miR-296* causes a decrease in cell growth. **Characterization of the molecular pairing between *miR-296* and *TSC2* currently has not been studied. The result will greatly impact our current understanding of whether *TSC2*, a tumor suppressor gene, can be regulated by miRNAs.**

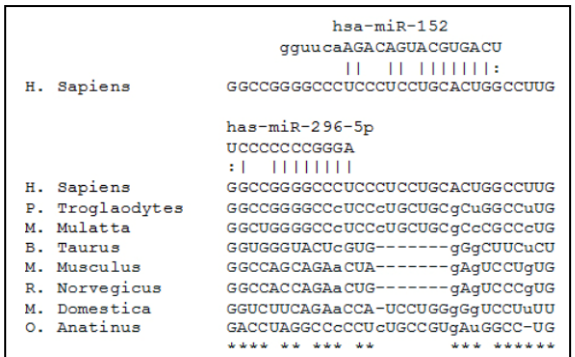

Figure 1 *miR-296* 'seed' sequence is completely matched with TSC2 3'UTR. This seed is evolutionarily conservative among species. In comparison to *miR-296*, the *miR-152* binding site is not conservative among species.

BODY

TSC2 plays a key role in the Akt-mTOR signaling pathway and is associated with many benign and malignant neoplasms. **The hypothesis is that *miR-296* is the negative regulator of TSC2 in some normal or tumor cells.** To explore whether TSC2 can be regulated by the endogenous miRNAs during development and tumorigenesis, the relationship between *miR-296* and TSC2 will be analyzed in two different approaches, both *in vitro* and *in vivo*.

Aim 1. To characterize whether *miR-296* is a native molecule that can specifically repress TSC2 expression *in vitro.* The molecular pairing of *miR-296*::*TSC2* will be tested on several different levels. First, it will be examined whether exogenous *miR-296* and its inhibitor can repress endogenous TSC2 expression. Next, it will be determined whether presence of the *TSC2* 3'UTR sequence is required for *miR-296*-mediated TSC2/luciferase activities. Finally, the correlation between TSC2, the mTOR pathway and cell proliferation in cells with constant overexpression of *miR-296* will be will be examined.

Aim 2. To examine the correlation between levels of endogenous TSC2 and endogenous *miR-296* expression *in vivo*. The levels of TSC2 and *miR-296* expression may be cell-type-specific; examination of these two molecules by in situ hybridization (ISH) and immunohistochemistry (IHC) will be conducted in adult normal tissues as well as the selected solid tumor tissues.

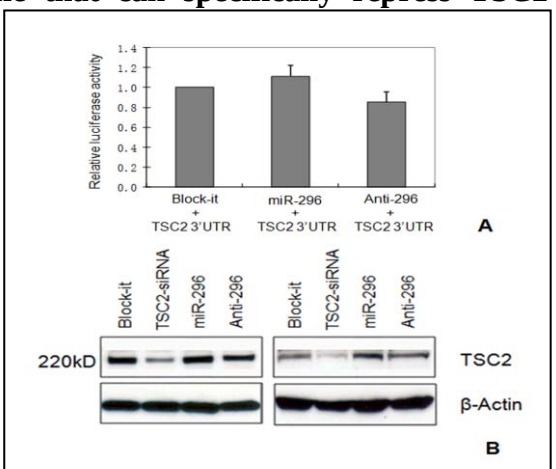

Fig 2 Expression analysis of the regulation role of *miR-296* for its predicted target gene *TSC2 in vitro*. A. Transient transfection analysis for luciferase expression with *TSC2* 3'UTR in the presence and absence of *miR-296*. B. Transient transfection analysis of miR-296 for TSC2 expression by Western blot analysis. *TSC2* siRNA was used as a positive control against for TSC 2 expression. Actin was used as protein loading control.

Key Research Accomplishments

Experiment 1. Transient transfection analysis of *miR-296* in HEK293T cell line. HEK293T cell line (which has high TSC2 and low *miR-296*, data not shown) were used for this study. Transfection were performed in Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells ($5x10^4$) were plated onto each of 12-well plates. To estimate transfection efficiency, the Block-iT (Fluorescent double-stranded random 22mer RNA from Invitrogen, Carlsbad, CA) were used as a negative control. HEK293T cells were transfected with 20, 40, 60 pmol of *miR-296* mimic (Dharmacon Inc., Lafayette, CO) in triplicate. Cells treated with the *miR-296* inhibitor were used as a control to block the effects of endogenous *miR-296*. Cells were harvested at 48 hours post-transfection and total RNA and proteins were prepared according to standard protocols, as described previously (6-8) The efficacy of transfection was validated by *mir*Vana qRT-PCR of *miR-296* and U6 (control) with the *mir*Vana qRT-PCR Kits (Ambion, Austin, TX). As seen in Figure 2B, by transient transfection of *TSC2* siRNA, significant reduction of TSC protein was noted.

In the same experiment, application of miR-296 mimic and inhibitor has minimal effect on TSC2 expression (not shown) and translation (Figure 2B). The experiment was repeated in different cell line showing the similar finding (Figure 2B right panel).

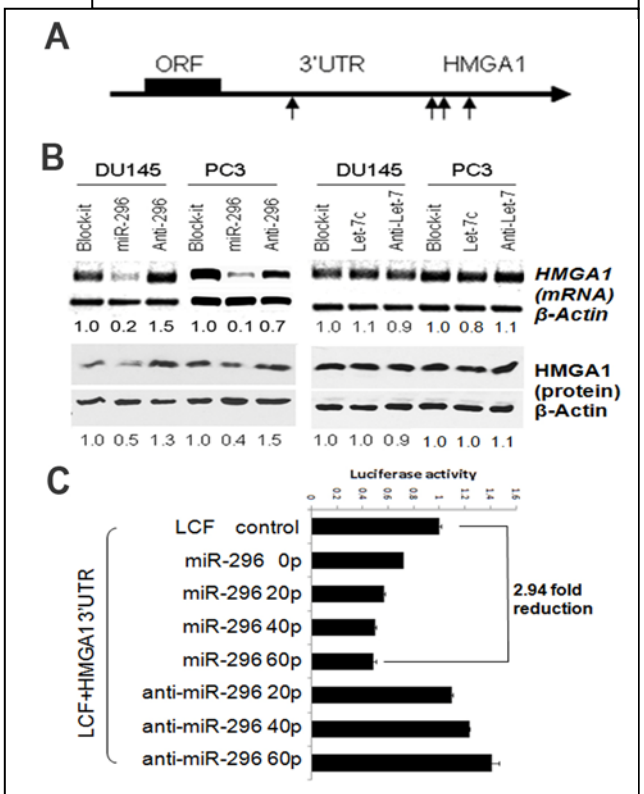

Figure 3. Repression of HMGA1 expression by *miR-296* in PC. A. Depiction illustrating four complementary sites of *miR-296* in 3'UTR of *HMGA1* gene. B. *miR-296* (left panel), but not *let-7c* (right panel), repression of HMGA1 expression at post-transcriptional (upper panel) and translational (lower panel) levels in PC3 and DU145 cell lines. C. Luciferase activity (x-axis) was shown in PC3 cells transfected with LCF vector only (channel 1), LCF plus *HMGA1* 3'UTR construct (with 3 CSs) and cotransfected with *miR-296* mimic (channel 2-5) and inhibitor (channel 6-8). Y axis represented the concentration of microRNAs in pmol.

Experiment 2. Luciferase expression analysis in the presence and absence of *TSC2* 3'UTR.

We previously found that *TSC2* was significantly down regulated in leiomyomas (9-10). Down regulation of *TSC2* was also found in this study. We found that miR-296 was inversely correlated with TSC2 protein in 36 leiomyomas.

TSC2 contains 41 exons with a very short 3' untranslation region (3'UTR, <110 nt). The short *TSC2* 3'UTR may prevent from miRNA regulation. However, in the *TSC2* 3'UTR immediately adjacent to the stop code, there is a highly conserved sequence that harbors the complementary sites of *miR-296* and a few other miRNAs (Figure 1). To study whether TSC2 is the target of *miR-296*, we prepared TSC2 3'UTR construct and examined the luciferase activity by treated cells with control, *miR-296* mimic and inhibitor.

PC3 cell line has moderate levels of *miR-296* and low levels of TSC2 expression (data not shown). Luciferase constructs of psiCHECK2-TSC2 3'UTR vectors containing a 110 bp fragment of the *TSC2* 3'UTR with or without putative *miR-296* complementary site were introduced into PC3 cells with the aid of Lipofectamine 2000. Luciferase activities were measured using the Dual-Glo™ Luciferase Assay System (Promega) following slightly modified manufacturer's instructions.

In triplicate cell lines treated with miR-296 mimic and inhibitor, there was no reduction of luciferase expression in cells (Figure 2A). The findings indicated that *TSC2* was not the direct target of *miR-296*. The inverse correlation of *TSC2::miR-296* pair may be related to some other molecular mechanisms in leiomyomas. This finding was published as part of our study in uterine leiomyoma in PloS One (11).

Experiment 3. Functional analysis of TSC-mTOR pathway by disrupting *miR-296*::TSC2 pairs in HEK293T cell line. Although computer analysis revealed that TSC2 was the predicted target gene (Figure 1), but we found that TSC2 was not regulated by *miR-296* (see experiment 1 and 2) in molecular level. It apparently was lack of basis for further analysis of how miR-296 mediated TSC dysregulation in mTOR pathway. To better use our system and resource to characterize the role of *miR-296* in regulation of other oncogenic factors, we turned our attention to a new target of miR-296: HMGA1. We established stable miR-296 cell lines in prostate cancer cell lines and examined and characterized that HMGA1 was the direct target of miR-296 (Figure 4). We further analyzed the functional role of miR-296 in association with HMGA1 mediated tumorigenesis in prostate cancer (Figure 5). The study was published in Clinical Cancer Research (12).

Experiment 4. *In situ* hybridization (ISH) analysis of *TSC2* cRNA and *miR-296* and Immunohistochemistry (IHC) analyses of tuberin in tissue microarray (TMA). Again, when we found miR-296 did not regulate TSC2, further comparison of miR-296 and TSC2 in the candidate tumors and normal tissues listed in the proposal will not yield the expected results. We therefore turn our research direction to more specific target of miR-296. We prepare human tumor samples which more relevant to our newly identified molecular pairs of miR-296 and HMGA1, including leiomyomata, prostate cancer and ovarian cancer. We collected and prepared high density tissue microarrays from 36 uterine leiomyomas (9-10, 13), 30 prostate cancer (unpublished data), 115 cases of ovarian cancer (6 different types) (14). A high-density TMA (0.6-1.0 mm tissue cores) were prepared by the MTA-1 manual tissue arrayer (BeecherInstruments, Sun Prairie, WI).

TMA blocks were sectioned at 4 microns. The detailed procedure for ISH followed the manufacturer's protocol. miRCURY LNA probes of *miR-296* (test) and *U6* (control) were purchased from Exiqon (Vedbaek, Denmark). The expression levels of *miR-296* were scored based on the intensity of ISH signals on each core by a density photometry (NIH Scion). IHC stains for TSC2 and HMGA1 (the tumorigenic factor for uterine leiomyoma and prostate cancer) were performed on a Ventana Nexus automated system (Tucson, Arizona) with antibodies of tuberin and other control markers (including hamartin, cytokeratin, vimentin and gFAP). Immunoreactivity were scored semiquantitatively based on intensity and percentage.

Correlation analysis Between *TSC2* protein tuberin, HMGA1 and *miR-296* were performed by a case matched analysis. Statistical significance was analyzed by a paired t-test. We found that there significant increase of *miR-296* in all types of tumors including leiomyomas, prostate cancer and ovarian cancer. In contrast, downregulation of TSC2 were found in the same groups of tumors. However, inverse correlation of TSC2 and *miR-296* were only found in leiomyomas, not in prostate cancer and ovarian cancer. In contrast, we found that HMGA1 was the direct target of miR-296 and these two paired molecules showed moderate correlation in prostate cancer (Figure 4) and leiomyoma (data not shown).

Reportable Outcomes

See above results and two publications listed below.

Conclusion

We found that TSC2 was downregulated in leiomyomata and prostate cancer. Although TSC2 was inversely correlated with miR-296 expression in leiomyomata, but this correlation could not be established in prostate cancer (data not shown). We found that miR-296 could not directly repress TSC2 expression based on expression analysis and luciferase analysis (Figure 2). This negative results force us to adjust our research direction into new miR-296 targets in association with solitary tumors. We would be able to identify HMGA1, a novel target of miR-296 in prostate cancer. Further infestation revealed that impairing of miR-296::HMGA1 expression contributed to prostate carcinogenesis.

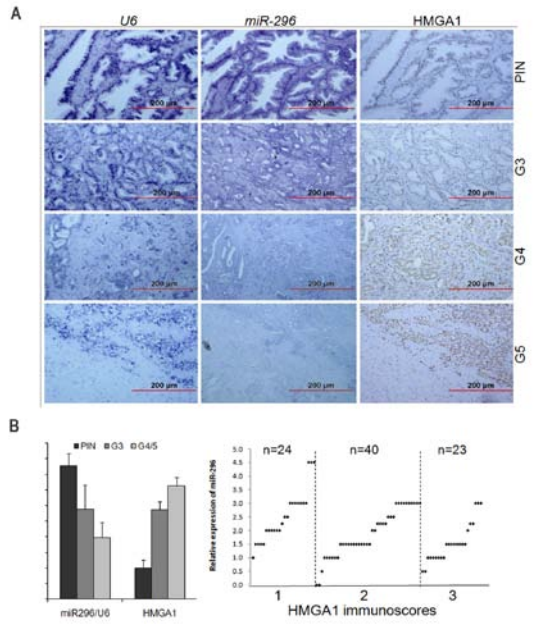

Figure 4. Correlation analysis of HMGA1 and *miR-296* expression in PC population. A. HMGA1 (IHC) and *miR-296* (ISH) expression in high grade prostate intraepithelial neoplasia (PIN), prostate carcinoma (Gleason score 3, 4 /5) PC tissue. *U6* was RNA control for *miR-296*. B. HMGA1 (right) and *miR-296/U6* (left) in PIN, low (G3) and high (G4-5) grade PC. C. Dot plot analysis of the relative expression of *miR-296/U6* in different immunointensity (scales 1-3) for HMGA1.

References

1. Jansen FE, Notenboom RG, Nellist M, Goedbloed MA, Halley DJ, de Graan PN, et al. Differential localization of hamartin and tuberin and increased S6 phosphorylation in a tuber. Neurology. 2004 Oct 12;63(7):1293-5.
2. Meikle L, McMullen JR, Sherwood MC, Lader AS, Walker V, Chan JA, et al. A mouse model of cardiac rhabdomyoma generated by loss of Tsc1 in ventricular myocytes. Hum Mol Genet. 2005 Feb 1;14(3):429-35.
3. Jozwiak J, Grajkowska W, Kotulska K, Jozwiak S, Zalewski W, Zajaczkowska A, et al. Brain tumor formation in tuberous sclerosis depends on Erk activation. Neuromolecular Med. 2007;9(2):117-27.
4. Niida Y, Stemmer-Rachamimov AO, Logrip M, Tapon D, Perez R, Kwiatkowski DJ, et al. Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions. Am J Hum Genet. 2001 Sep;69(3):493-503.
5. Jozwiak J, Jozwiak S, Wlodarski P. Possible mechanisms of disease development in tuberous sclerosis. Lancet Oncol. 2008 Jan;9(1):73-9.
6. Shi G, Perle MaA, Mittal K, Chen H, Zou X, Hernando E, et al. Let-7 repression leads to HMGA2 overexpression in uterine leiomyosarcoma. J Cell Mol Med. 2009;online Feb 24.
7. Peng Y, Laser J, Shi G, Mittal K, Melamed J, Lee P, et al. Antiproliferative effects by Let-7 repression of high-mobility group A2 in uterine leiomyoma. Mol Cancer Res. 2008 Apr;6(4):663-73.
8. Wang T, Zhang X, Obijuru L, Laser J, Aris V, Lee P, et al. A micro-RNA signature associated with race, tumor size, and target gene activity in human uterine leiomyomas. Genes Chromosomes Cancer. 2007 Apr;46(4):336-47.
9. Wei J, Chiriboga L, Mizuguchi M, Yee H, Mittal K. Expression profile of tuberin and some potential tumorigenic factors in 60 patients with uterine leiomyomata. Mod Pathol. 2005 Feb;18(2):179-88.
10. Wei JJ, Chiriboga L, Arslan AA, Melamed J, Yee H, Mittal K. Ethnic differences in expression of the dysregulated proteins in uterine leiomyomata. Hum Reprod. 2005 Sep 19.
11. Zavadil J, Ye H, Liu Z, Wu J, Lee P, Hernando E, et al. Profiling and functional analyses of microRNAs and their target gene products in human uterine leiomyomas. PLoS One. 2010;5(8):e12362.
12. Wei JJ, Wu X, Peng Y, Shi G, Olca B, Yang X, et al. Regulation of HMGA1 expression by microRNA-296 affects prostate cancer growth and invasion. Clin Cancer Res. 2011 Mar 15;17(6):1297-305.
13. Wei JJ, Chiriboga L, Mittal K. Expression profile of the tumorigenic factors associated with tumor size and sex steroid hormone status in uterine leiomyomata. Fertil Steril. 2005 Aug;84(2):474-84.
14. Wei JJ, Wu J, Luan C, Yeldandi A, Lee P, Keh P, et al. HMGA2: a potential biomarker complement to P53 for detection of early-stage high-grade papillary serous carcinoma in fallopian tubes. Am J Surg Pathol. 2010 Jan;34(1):18-26.

Appendices

Two manuscripts related to this study have been published listed below.

1. Wei JJ, Wu X, Peng Y, Shi G, Olca B, Yang X, et al. Regulation of HMGA1 expression by microRNA-296 affects prostate cancer growth and invasion. Clin Cancer Res. 2011 Mar 15;17(6):1297-305.

2. Zavadil J, Ye H, Liu Z, Wu J, Lee P, Hernando E, et al. Profiling and functional analyses of microRNAs and their target gene products in human uterine leiomyomas. PLoS One. 2010;5(8):e12362.